FURTHER STUDIES ON THE RESOLUTION OF FUNGAL CELLULASE BY ZONE ELECTROPHORESIS

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The demonstration by starch block zone electrophoresis of the occurrence of as many as eight electrophoretically distinct, cellulolytically active components in a fungal cellulase preparation[1] raised questions as to whether more refined techniques might reveal additional components or to what degree the observed results might represent an artifact. Studies designed to examine these questions constitute the subject of the present paper. These studies include (1) effect of size of sections into which the starch block is cut, (2) shape and uniformity of zones across the block, (3) reproducibility of enzyme activity measurements, (4) effect of nature of supporting medium, and (5) effect of source of cellulase. To further test the reasonableness of the occurrence of multiple components in cellulase, an attempt was made to construct a hypothetical electrophoretic diagram which was similar to those observed experimentally.

MATERIALS AND METHODS

Cellulase preparations from three different species of fungi were studied. The preparation most studied originated from a lyophilized stock obtained from a cell-free culture filtrate of *Myrothecium verrucaria* QM 460 which had been grown on cellulose in liquid medium[1]. The other preparations, supplied by E. T. REESE of these laboratories, consisted of an aqueous solution of an acetone precipitate of a filtrate of a shake culture of Basidiomycete QM 806 grown on ground cellulose and an original filtrate of a still culture of *Penicillium pusillum* QM 137g grown on cotton duck strips.

The zone electrophoresis was carried out with the aid of starch blocks 100 × 3.3 × 0.3 cm in size according to procedures described elsewhere[2,3]. The conditions of ionic strength and field strength which were used were based on the results of studies with known test materials[3]. The buffer was 0.1 ionic strength sodium phosphate at pH 7, the field 8 V/cm, and the duration of the runs 40 h.

The test samples consisted of 0.1 ml aliquots of solution of cellulase containing around 100 units of the enzyme. Enzyme activities of the test samples were determined by a modification of the method of REESE, SIU AND LEVINSON[4]. For convenience, however, data for the distribution of enzyme activity in the zone electrophoretic experiments were recorded directly as arbitrary units of absorbance of the colored

solutions produced in the tests, without conversion to units of activity. Dextran was used as a marker in certain of the tests. It was measured by the method of RIMINGTON[6].

EXPERIMENTAL

Size of sections into which block is cut

When starch blocks at the ends of runs were cut into 1-cm pieces and the pieces were extracted with water and analyzed for enzyme activity, the results were very similar to those reported previously, using a starch block of different dimensions[1]. Nine fairly distinct components could be seen, although no one of them was completely separated

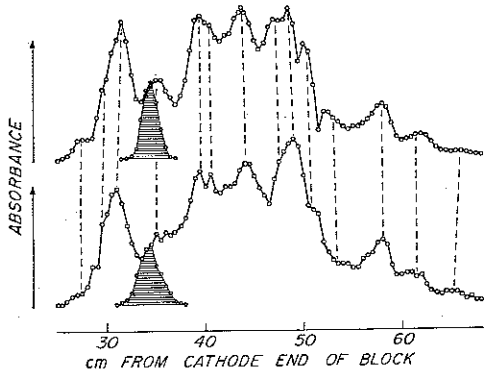

Fig. 1. Electrophoretic diagrams of *Myrothecium verrucaria* cellulase for blocks cut into 0.5-cm sections. Upper diagram, test 1; lower diagram, test 2.

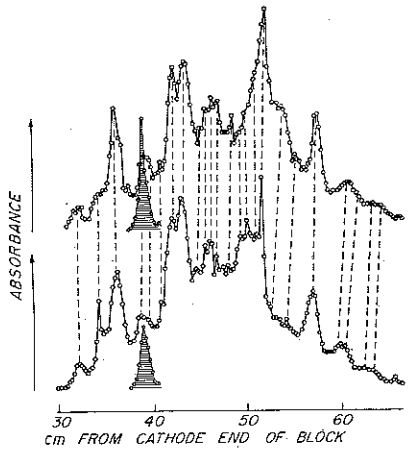

Fig. 2. Electrophoretic diagrams of *Myrothecium verrucaria* cellulase for blocks cut into 0.25-cm sections. Upper diagram, test 1; lower diagram, test 2.

from all of the others. When the blocks were cut into 0.5-cm sections, however, five further components were revealed to give a total of fourteen. This is shown by the diagrams in Fig. 1 for two different tests. The dotted lines are drawn to show correspondence between components. The shaded peaks represent dextran used as a marker. When the blocks were cut into 0.25-cm sections, further components were revealed to give a new total of at least twenty-four, shown by the diagrams in Fig. 2.

The significance of the multiplicity of components revealed in the foregoing tests was supported by the reproducibility of the results, a total of fourteen tests having been made with blocks cut into 1-cm sections, three tests with blocks cut into 0.5-cm sections, and four tests with blocks cut into 0.25-cm sections. It was also supported by the results of a large number of additional tests not reported in this paper in which ionic strength, voltage, and mode of application of sample were varied[6]. The degree to which heights and positions of peaks varied in the different runs was probably due to slight variations in techniques for preparing the starch blocks and for analyzing the fractions which were instituted from time to time as the work progressed.

Shape and uniformity of zones across the block

To determine whether curvature or cometing of zones occurred under the conditions of electrophoresis used and whether such behavior might to some degree be responsible for the appearance of multiple components in cellulase, experiments were carried out in which the blocks at the ends of the runs were, in effect, cut into six 100-cm long strips as well as into 0.5-cm transverse sections. The diagrams which were obtained for the first three of the six strips are shown in Fig. 3. Those obtained for the remaining three strips, not shown, were found to be very similar. It can be seen that matching of peaks was generally very good, indicating that the curvature of the zones was too slight to be a significant cause of artifacts.

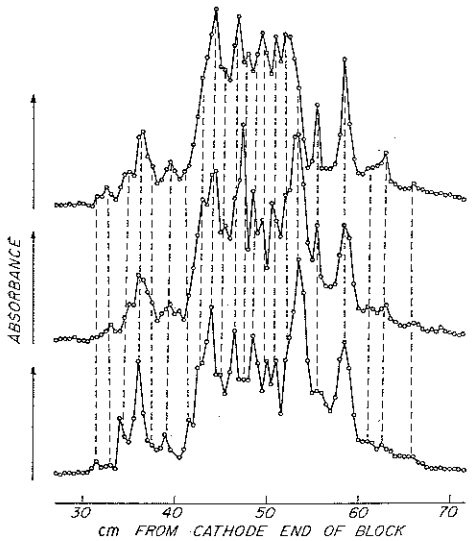

Fig. 3. Electrophoretic diagrams of *Myrothecium verrucaria* cellulase for 3 of 6 parallel strips from block cut into 0.5-cm sections.

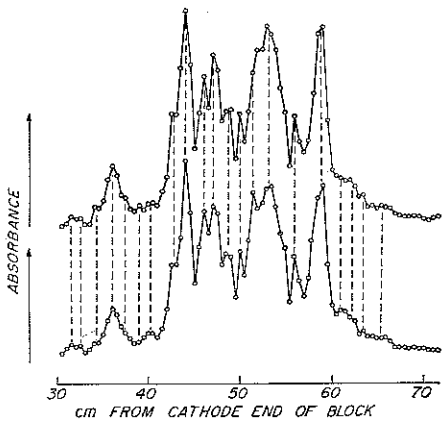

Fig. 4. Comparison of results of measurements of cellulase activity made on two different days.

Reproducibility of enzyme activity measurements

To determine whether errors arising from enzyme activity measurements were likely to cause the appearance of spurious components, extracts taken from the fifth strip of the starch block of the preceding test were analyzed for enzyme activity on two different days. The results, shown in Fig. 4, demonstrate that matching of peaks was highly adequate in every instance and that, although exact reproducibility of individual data was not attained, in few cases did peaks appear or disappear due to errors in activity measurement.

Nature of supporting medium

Earlier work indicated that cellulase was not adsorbed to any detectable degree by the starch used as a supporting medium[1]. Because of the possibility, however, that even a slight affinity of cellulase for starch might give rise to peculiar chromatographic

effects manifesting themselves in the form of multiple components, zone electrophoresis experiments were carried out with other types of supporting media. It was not to be expected that different supporting media would show similar chromatographic effects, assuming that such effects could exist. Blocks made up with glass beads[7], powdered polyvinyl chloride Geon-426[8], and powdered nylon were tried. Results with the glass beads were unsatisfactory because of excessively high electro-endosmotic rates. With the polyvinyl chloride the endosmosis was also quite high, but this could be reduced by placing several layers of cellophane dialysis membrane over the ends of the block. Patterns of enzyme activity obtained when either polyvinyl chloride or powdered nylon were used as supporting media were strongly reminiscent of those obtained when the starch granules were used, but the different cellulolytic components were observed to be much less well resolved. The dextran peaks obtained in these tests also were found to be abnormally diffuse, indicating that electrical or mechanical disturbances within the block had partially counteracted the resolution of the enzyme components. Nevertheless, the results suggest that the resolution of components observed with starch as the supporting medium are not attributable solely to specific chromatographic or other types of interaction between the enzyme and the supporting medium.

In an effort to exclude adsorption of enzyme on starch granules through the mass action of another protein, as suggested by MICHL[9], an experiment was carried out in which the 0.1 ionic phosphate buffer medium in the starch block contained 2 mg of hemoglobin per ml. The pattern of enzyme activity which was obtained under these conditions was almost identical with those already shown in Figs. 1 and 2 in which

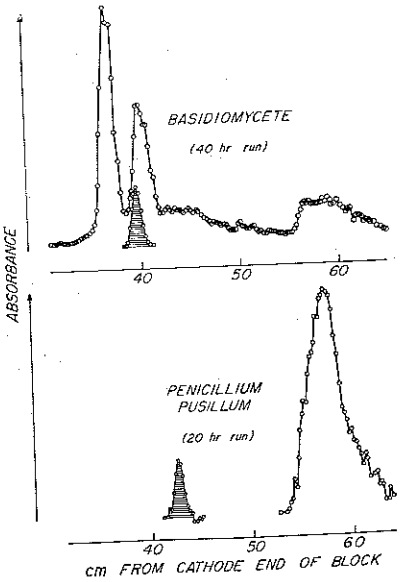

Fig. 5. Electrophoretic diagrams of cellulases from Basidiomycete and *Penicillium pusillum*.

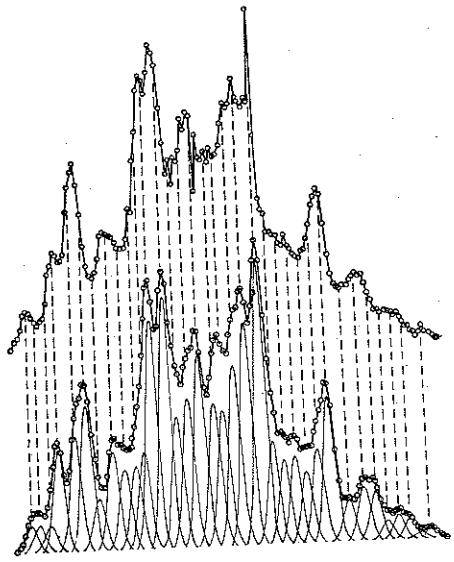

Fig. 6. Comparison of hypothetical and experimental electrophoretic diagrams. Upper diagram, experimental; lower diagram, hypothetical.

hemoglobin was not present, thus providing additional evidence that the appearance of multiple components was not the consequence of chromatographic effects.

Cellulase preparations from different fungi

Patterns obtained for cellulases from two organisms other than *Myrothecium verrucaria* are shown in Fig. 5. It appears possible, although not certain, that some of the components of the cellulases from *Myrothecium verrucaria* and the Basidiomycete are qualitatively identical, based on similarities in distances of migration. On the other hand, the components present in the cellulase from *Penicillium pusillum* appear to migrate altogether faster than those of the other fungi. Since the presence of artifacts might be expected to result in a similarity of patterns of cellulases from different sources, the observed difference in patterns can be taken as evidence for the absence of artifacts.

Construction of hypothetical electrophoretic diagram

It might be questioned whether under the conditions used it is possible to obtain the observed degree of resolution for such a large number of components. This question was tested by constructing an electrophoretic diagram consisting of a hypothetical mixture of 35 components, migrating at rates such that the centers of their zones would be 0.75 to 1.25 cm apart, having band widths comparable to that shown by dextran[3], and possessing selected enzyme activities. The result obtained is shown in the lower pattern of Fig. 6. The similarity of the hypothetical pattern to an experimental pattern shown above it indicates that the observed resolution is reasonable.

DISCUSSION

Although the possibility of an enzyme preparation containing as many as 24 or more physically different components appears exceptional, the phenomenon of a multiple-component enzyme is in itself by no means without precedent since it has already been demonstrated for ribonuclease[10–16], glyceraldehyde-3-phosphate dehydrogenase[17], invertase[18], lysozyme[19,20], amylase[21], peroxidase[22], trypsin[23,24], protease[25,26], chymotrypsin[27,28], chymotrypsinogen[28], β-galactosidase[29], phosphatase[30], esterase[21,26,31], acid phosphomonoesterase[32], phosphodiesterase[33], enolase[34], maltozymase[35], lactic acid dehydrogenase[36,37], laccase[38], hemolytic enzyme[39], and aryl-β-glucosidase[40]. As many as ten components have been detected in mouse liver esterase[31]. Particularly relevant is the observation in certain of the studies that the individual members of multi-component systems have differing substrate specificities[30,35].

There is also considerable evidence in addition to the present which indicates the existence of multiple components in cellulase preparations[40–44], although some evidence to the contrary has been presented [45–47].The probability, suggested by work of GILLIGAN AND REESE[41], GRIMES, DUNCAN AND HOPPERT[42], and HASH AND KING[40], that the different components of fungal cellulase possess differing specificities with respect to cellulose in its different forms or stages of degradation merits further investigation. The isolation and study of pure individual components should provide definitive answers to the question.

SUMMARY

Refinement in technique has served to show the presence of twenty-four or more electrophoretically distinct, cellulolytically active components in a fungal cellulase preparation. Studies of uniformity of widths of zones, reproducibility of activity measurements, nature of supporting medium, source of cellulase, and construction of hypothetical electrophoretic diagrams support the findings.

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